# Transcriptional Analysis of Four Family 4 P450s in a Puerto Rico Strain of *Aedes aegypti* (Diptera: Culicidae) Compared With an Orlando Strain and Their Possible Functional Roles in Permethrin Resistance

WILLIAM R. REID,[1] ANNE THORNTON,[2,3] JULIA W. PRIDGEON,[4,5,6] JAMES J. BECNEL,[4] FANG TANG,[1,7] ALDEN ESTEP,[2,4] GARY G. CLARK,[4] SANDRA ALLAN,[4] AND NANNAN LIU[1]

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**ABSTRACT** A field strain of *Aedes aegypti* (L.) was collected from Puerto Rico in October 2008. Based on $LD_{50}$ values by topical application, the Puerto Rico strain was 73-fold resistant to permethrin compared with a susceptible Orlando strain. In the presence of piperonyl butoxide, the resistance of Puerto Rico strain of *Ae. aegypti* was reduced to 15-fold, suggesting that cytochrome P450-mediated detoxification is involved in the resistance of the Puerto Rico strain to permethrin. To determine the cytochrome P450s that might play a role in the resistance to permethrin, the transcriptional levels of 164 cytochrome P450 genes in the Puerto Rico strain were compared with that in the Orlando strain. Of the 164 cytochrome P450s, 33 were significantly ($P < 0.05$) up-regulated, including cytochrome P450s in families four, six, and nine. Multiple studies have investigated the functionality of family six and nine cytochrome P450s, therefore, we focused on the up-regulated family 4 cytochrome P450s. To determine whether up-regulation of the four cytochrome P450s had any functional role in permethrin resistance, transgenic *Drosophila melanogaster* Meigen lines overexpressing the four family 4 P450 genes were generated, and their ability to survive exposure to permethrin was evaluated. When exposed to 5 μg per vial permethrin, transgenic *D. melanogaster* expressing *CYP4D24*, *CYP4H29*, *CYP4J15v1*, and *CYP4H33* had a survival rate of 60.0 ± 6.7, 29.0 ± 4.4, 64.4 ± 9.7, and 11.0 ± 4.4%, respectively. However, none of the control flies survived the permethrin exposure at the same concentration. Similarly, none of the transgenic *D. melanogaster* expressing *CYP4J15v1* or *CYP4H33* ?5 survived when they were exposed to permethrin at 10 μg per vial. However, transgenic *D. melanogaster* expressing *CYP4D24* and *CYP4H29* had a survival rate of 37.8 ± 4.4 and 2.2 ± 2.2%, respectively. Taken together, our results suggest that *CYP4D24* might play an important role in cytochrome P450-mediated resistance to permethrin.

**KEY WORDS** *Aedes aegytpi*, permethrin, resistance, cytochrome P450, detoxification

The yellowfever mosquito, *Aedes aegypti* (L.), is a globally distributed mosquito and the major vector of dengue virus (Gubler 1988, Warren and Mahmoud 1990, Gubler and Clark 1995). Management of *Ae. aegypti* is primarily through the use of chemical insecticides, of which pyrethroids were frequently used because of their low mammalian toxicity and high efficacy (Hougard et al. 2002, Juntarajumnong et al. 2012, Manda et al. 2013). However, frequent use of insecticides has led to the development of insecticide resistance in field mosquitoes (Hemingway et al. 2004, Ffrench-Constant et al. 2004, Liu 2008), making the management of *Ae. aegypti* populations problematic as higher doses of insecticide are needed to obtain the same level of control, ultimately leading to control failure (Vulule et al. 1994, Curtis et al. 1998, Liu 2008).

Insecticide resistance is a multifaceted phenomenon involving several mechanisms, including target site insensitivity, reduced penetration rate, and metabolic detoxification. In the case of metabolic detoxification, three major classes of enzymes are involved: cytochrome P450s, hydrolases, and glutathione-S-transferases (Feyereisen 1995, Ffrench-Constant et al. 2004, Hemingway et al. 2004, Yang and Liu 2011, Reid et al. 2012, Gong et al. 2013). Of the three detoxification enzymes, the role of cytochrome P450s in *Ae.*

[1] Department of Entomology and Plant Pathology, Auburn University, 350 South College Street, Auburn, AL 36849.
[2] Navy Entomology Center of Excellence, 937 Child Street, Jacksonville, FL, 32312.
[3] Current address: Department of Medicine and Department of Genome Sciences, University of Washington, Foege Building S-250, Box 355065, 3720 15th Ave., NE, Seattle WA 98195-5065.
[4] USDA–ARS, Mosquito and Fly Research Unit, Center for Medical, Agricultural, and Veterinary Entomology, 1600 SW23rd Dr., Gainesville, FL 32682.
[5] Current address: USDA, ARS, Aquatic Animal Health Research Unit, 990 Wire Rd., Auburn, AL 36832.
[6] Corresponding author, e-mail: Julia.pridgeon@ars.usda.gov.
[7] Current address: Institute of Vegetables and Flowers, Chinese Academy of Agricultural Science, 12 Zhongguancun Nandajie, Beijing 100081, China.

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14. ABSTRACT

**A ?eld strain of Aedes aegypti (L.) was collected from Puerto Rico in October 2008. Based onLD50 values by topical application, the Puerto Rico strain was 73-fold resistant to permethrin compared with a susceptible Orlando strain. In the presence of piperonyl butoxide, the resistance of Puerto Rico strain of Ae. aegypti was reduced to 15-fold, suggesting that cytochrome P450-mediated detoxi?cation is involved in the resistance of the Puerto Rico strain to permethrin. To determine the cytochrome P450s that might play a role in the resistance to permethrin, the transcriptional levels of 164 cytochrome P450 genes in the Puerto Rico strain were compared with that in the Orlando strain. Of the 164 cytochrome P450s, 33 were signi?cantly (P 0.05) up-regulated, including cytochrome P450s in families four, six, and nine. Multiple studies have investigated the functionality of family six and nine cytochrome P450s, therefore, we focused on the up-regulated family 4 cytochrome P450s. To determine whether up-regulation of the four cytochrome P450s had any functional role in permethrin resistance, transgenic Drosophila melanogaster Meigen lines overexpressing the four family 4 P450 genes were generated, and their ability to survive exposure to permethrin was evaluated. When exposed to 5 g per vial permethrin, transgenic D. melanogaster expressing CYP4D24, CYP4H29 CYP4J15v1, and CYP4H33 had a survival rate of 60.0 6.7, 29.0 4.4, 64.4 9.7, and 11.0 4.4% respectively. However, none of the control ?ies survived the permethrin exposure at the same concentration. Similarly, none of the transgenic D. melanogaster expressing CYP4J15v1 or CYP4H33 ?5 survived when they were exposed to permethrin at 10 g per vial. However, transgenic D. melanogaster expressing CYP4D24 and CYP4H29 had a survival rate of 37.8 4.4 and 2.2 2.2% respectively. Taken together, our results suggest that CYP4D24 might play an important role in cytochrome P450-mediated resistance to permethrin.**

15. SUBJECT TERMS

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**Table 1. List of primers used for the qPCR and the generation of constructs for the functional testing in transgenic *D. melanogaster***

| Primer | Gene[a] | Vectorbase[b] | Forward primer (5′–3′) | Reverse primer (5′–3′) |
|---|---|---|---|---|
| qPCR | CYP15B1 | AAEL002067 | CGGATTCGTTCCTTCGATAA | ATGGAATTCAGCACCGAAAC |
| | CYP18A1 | AAEL004870 | CAGTGAAGGTCAGCTGTGGA | CGAGACGGAGAGGTACTTGC |
| | CYP303A1ae | AAEL012144 | GATAGCACGAGCACGACAAA | CCAAGTCCGGGTTTCATAGA |
| | CYP304B2xx/yy | AAEL014412 | GATTGGAAGGAGCAGAGACG | CCTTTCACCGGTTAGCACAT |
| | CYP304B3yy/xx | AAEL014411 | GGTCAGTTCTACCCGACCAA | TCAAATGCCTCAGCACAAAG |
| | CYP304C1 | AAEL014413 | GGGAGAATCTACCGGAAAGG | CTCGCGGTACATTTTGGTTT |
| | CYP305A6 | AAEL002071 | GCTCCCATTCTTCGGTAACA | TTCCCAATCTGGTTCCCATA |
| | CYP305A5 | AAEL002043 | AGCCCTCTCCAAGCAGTACA | AGCCTTGTCCCCATAGTCCT |
| | CYP306A1 | AAEL004888 | TCGTGTGATATCCGCAATGT | GCGAGGTTAGTCAGCGTTTC |
| | CYP307A1 | AAEL009762 | GCCCTGCTGAAAAGTCTACG | GCCTTTGTCGGTAACGTGAT |
| | CYP307A1 | AAEL009768 | GAGTCAGCCTCAGGAAGTGG | CCCGTTCTGATTCAACACCT |
| | CYP307B1 | AAEL006875 | ATCATGGAAGCGCTGAGACT | GGTTCTCCCAGAGGTTCTCC |
| | CYP6F2 | AAEL014678 | CGTGAGTGCACCCAAGACTA | GAGCGTACGGTTTGCTCTTC |
| | CYP6F3 | AAEL014684 | GTGGCGTTTGGCATTAAGAT | CCGTACGACACCGTTTTTCT |
| | CYP6M5 | AAEL009117 | TGGATCTGCTGATCGCTATG | AGCACTTCTTGGACGCATTT |
| | CYP6M6 | AAEL009128 | AGAAAATACCCACCCGTTCC | GGTCGAATCTTTTCGGATCA |
| | CYP6M10 | AAEL009125 | TGAGTGCAACTCGATGAAGG | ATTCCACCGTGCTTTTTACG |
| | CYP6M11 | AAEL009127 | TTGTTCACGACAGGCAGAAG | CCTCGCTGCTTTTATTCTCG |
| | CYP6N6 | AAEL009126 | TTCTTCATCGGTGCTGTGAG | TTTGCGCAAGTTCGTACAAG |
| | CYP6N9 | AAEL009121 | ACCGCAACCCAAGACTACAC | AAACGCATCCCGATACAGAC |
| | CYP6N11 | AAEL009119 | CTGCCTGTCTACGCAATTCA | CAAAATTTTCAACGGGCTTG |
| | CYP6N11 | AAEL009138 | TGGGTTATCTGGCGCTATTC | ACACATCCTTGGCAAAGTCC |
| | CYP6N12 | AAEL009124 | TTCACTTGCGCGATCACTAC | TGCAGCAATTTCCTCAACAG |
| | CYP6N13 | AAEL009137 | ACAATGTCCCGAACTCGAAC | CATCATTCCGAATCGTGTTG |
| | CYP6N14 | AAEL009133 | ACGTTTTCTTCCGGGAAACT | TTTCGCCCATTTTTCTGAAC |
| | CYP6N15 | AAEL009122 | GTCAAGGCGTACCGTTCATT | CGCGATCGTGAAAGTACTGA |
| | CYP6N16 | AAEL010151 | AAAACCTCGCAAGAAAAGCA | GCCACCACCTCGTTCATACT |
| | CYP6N17 | AAEL010158 | AAGCATCACCCAGAACCAAC | ACCTTCTTGGCCAGGTTCTT |
| | CYP6P12v1 | AAEL012491 | GGCAGTTTTTGGTGGACAGT | CTGCTCGAACACCTTCTTCC |
| | CYP6S3 | AAEL009120 | AGGCAGATGGGGAAGAGAAT | TCAACAGCTGCATGAAGTCC |
| | CYP6Y3 | AAEL009132 | GCTAGTGGCTGCCGTTCTAC | CAGAGGCGAAGTCAACATGA |
| | CYP6Z6 | AAEL009123 | CGAGGTGTCTACTGCAACGA | TAACTTGTGCCCAACATCCA |
| | CYP6Z7 | AAEL009130 | GAGATCCGTTTTCTGCAAGC | GGTTCGCGATTCTCAGCTAC |
| | CYP6Z8 | AAEL009131 | CCTGAGATGATCCGATTCGT | CTCTTCGAAACCCAAAGCTG |
| | CYP6Z9 | AAEL009129 | TCCAATGGAGCAATCACGTA | ATCGTTCCGGAATGTAGCAC |
| | CYP6AA5v1 | AAEL012492 | CCAGCTTCGAGCCTTTTATG | TGAAGACTCTGTCGGCAATG |
| | CYP6AG3 | AAEL007024 | CCGAACGTTTTAACCCAGAA | CTCGTTTGCCCGAAAAGTAGC |
| | CYP6AG5 | AAEL006984 | ACATTCCGCAGAAGATGGTC | TAGGTGGATAGCCCAGATCG |
| | CYP6AG6 | AAEL006992 | ACACCCGCGTTTACTACGTC | GCGCATCACAAGCAAAGATA |
| | CYP6AG7 | AAEL006989 | TGTTTCCAGCTGCATCTTTG | TTTCGCTTGCATCACGATAG |
| | CYP6AG8 | AAEL003890 | ACCAGCACACGGAAAGTACC | AACCTGCATACGACCGAAAC |
| | CYP6AH1 | AAEL007473 | CTGCGTGCTGAAAGAGTACG | ATCCGCTTACCAACGTCATC |
| | CYP6AH1 | AAEL015641 | CTGCGTGCTGAAAGAGTACG | ATCCGCTTACCAACGTCATC |
| | CYP6AK1 | AAEL004941 | AAGGATTGTACGCCGATTTG | CTCAAGGAATCCGGTTACGA |
| | CYP6AL1 | AAEL008889 | AACCGAGAATGCACCAAAAC | CGACTGTTCGTCACTGGAGA |
| | CYP6AL3 | AAEL009656 | GGCAAAGGTTCATCAGGAAA | CACTATGGGTGTGCCCTTCT |
| | CYP6BB2 | AAEL014893 | TAGTCGCTAAGGACGGAGGA | AAGTACTCCGGATCGTGGTG |
| | CYP6BZ1 | AAEL012494 | GTAGGGCAAATGCTGTGGAT | ACTCCGTTGAACGTTGTTCC |
| | CYP6CA1 | AAEL014680 | TCGAGGGACTTTCAGCACTT | AACAAATCGGCCACGTCTAC |
| | CYP6CB1 | AAEL002046 | TAAGCAGCGCACCTCCTATT | AAAATCAACGGTCAGCATCC |
| | CYP6CB1 | AAEL009018 | GAGTGCAACAGCATGAGGAA | GTCATCGTCGTTCAGCTTCA |
| | CYP6CB2 | AAEL002872 | GTTTCGGAGATGGTCCAAGA | GGTTGAAGCATCAGCAGTGA |
| | CYP6CC1v1 | AAEL014890 | GGGAACAGTTGGCAGGATAA | AAGTCCGTGTTTGGGTCTTG |
| | CYP6CD1 | AAEL005006 | TGGCCATTCTCTAGCGTTCT | TGCAGGTGGTGTAATCCGTA |
| | CYP9J2 | AAEL006805 | ACCGTTACGCCAACAAAGAC | GACGATTTTCGATCGGTTGT |
| | CYP9J6 | AAEL002638 | CAGCGTCAAACCAAGGGTAT | GTTTCAAATCCAGCCAGGAA |
| | CYP9J7 | AAEL014606 | CGGATATGGTGCACTTGTTG | GGTTCAACGCCAGTTCGTAT |
| | CYP9J8 | AAEL006811 | CCTCAACCGCAAGTACCAAT | GTCCTTGACACCGATTTGCT |
| | CYP9J9 | AAEL006793 | TGATCGCTCAGTGTTTCCTG | TTCGTAGGTCAATGGCTTCC |
| | CYP9J9 | AAEL014605 | TGATCGCTCAGTGTTTCCTG | TTCGTAGGTCAATGGCTTCC |
| | CYP9J10 | AAEL006798 | TATGGCGGAGTTTTTCAAGG | CACCGATAGCGATTGGAAGT |
| | CYP9J15 | AAEL006795 | GTACTACCCACAGCCGGAAA | ACACAACCGCTTTCATCTCC |
| | CYP9J16 | AAEL006815 | ACGATTGCCATACACAACGA | TCTGCGTCTTCTCCGTAGGT |
| | CYP9J17 | AAEL009699 | GGAGAAATTGGGGGTTGATT | TCATCCATCTCGTGCTTCAG |
| | CYP9J17 | AAEL006784 | GAAAGGGAGCACTGAAGCAC | AGGTCAAACCCGTAGACACG |
| | CYP9J18v1 | AAEL006804 | TCCCAGATCCAGATCGTTTC | AATTTGCGTCTTTTCCGTTG |
| | CYP9J19 | AAEL006810 | CCAACCTTTCTCGTTGGAAA | CTTCTACGGGTTGGTCGGTA |
| | CYP9J19 | AAEL014611 | CATCCAGAACGATCCGAAGT | GTCCGCTCCAGACTGAACTC |
| | CYP9J20v1 | AAEL006814 | ACGGAACGACATGATCAACA | AGCAACTCGTAGGCCAAGAA |
| | CYP9J20v2 | AAEL014604 | TGAGGTTGATGTTCGAGCTG | CCCATTGGTGAAGAACTCGT |
| | CYP9J21 | AAEL014612 | GTACAGCGTCTTTCCGAAGC | TGCATCAACAGGTGGATCAT |

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**Table 1. Continued**

| Primer | Gene[a] | Vectorbase[b] | Forward primer (5′–3′) | Reverse primer (5′–3′) |
|---|---|---|---|---|
| | CYP9J22 | AAEL006802 | TGTTGATGCAGGCAAAGAAG | CTGCCAGGAAAAAGACGAAG |
| | CYP9J23 | AAEL014615 | GTGCACCTTTCCGGAGTTTA | AAGGGTCTTCTCGAACAGCA |
| | CYP9J24 | AAEL014613 | TTCCCAACGTATGCGTTACA | GAGGAACTTCCGTCTTGCTG |
| | CYP9J26 | AAEL014609 | AGATGATCGCACAGTGCTTG | GGGCCACATTCTCAGTGTTT |
| | CYP9J27 | AAEL014616 | ACGGCAAGAAAATGATGGAC | CGGTTCCATGACTCTCCCTA |
| | CYP9J27 | AAEL014607 | CGGCACGGTAAGAAAATGAT | AGCCTTGATCGTCTCCTGAA |
| | CYP9J28 | AAEL014617 | TTTCCTCGACAAACCGATTC | AAAGTCCTTAACGGCCACCT |
| | CYP9J29 | AAEL014610 | GATGAGCAGCACAGGTCAAA | AGTGGATTGCCCATTTGAAG |
| | CYP9J30 | AAEL014603 | TCAAAGTCCTCGGGATGTTC | ATATTACGCCATCGCTGACC |
| | CYP9J31 | AAEL002633 | TTTTCAGCGATTGAGTGTCG | ATATCCTTGGTCTGGCGTTG |
| | CYP9J32 | AAEL008846 | GCCGTGACTACGTTTTGGAT | CTCGATCCAATGCAATTCCT |
| | CYP9M4 | AAEL001320 | GGTTGATCACGAAGGACGTT | AACCGTCGTAAATCCAGCAC |
| | CYP9M5 | AAEL001288 | GTCGGTTCTCAGCTTCGTTC | ATGGTCCTCGAACGTGTAGG |
| | CYP9M6 | AAEL001312 | CAGTGGCCACCTACGATTTT | CGCTGGATCGTGGATAAGAT |
| | CYP9M7 | AAEL001292 | AGGACTATGGCCGGTTTTCT | GCCCTAGAATCGGATCAACA |
| | CYP9M8 | AAEL009591 | AGCTGCCACTGTAGCCACTT | TTTGCTGCATCGATTCGTAG |
| | CYP9M9 | AAEL001807 | CACTAAGGAAATGGCCTCCA | TCCGGATCAAATCTTTCTGG |
| | CYP9AE1 | AAEL003748 | CAGTTCGGGATGGAGTTGTT | TCAACTCGTCGTCACTCCAG |
| | CYP329B1 | AAEL003763 | CTTCCTGGACGAAAGCAAAG | TAGTTCCGAATCCACGGAAG |
| | CYP4C38 | AAEL012266 | GAAAAGTCCCACGGCTATGA | CTTTCGTGATGAAGGGGAAA |
| | CYP4C50 | AAEL008017 | AAATTCGGAAGCAGAAGCAA | ATGCCTCGATCAACAGATCC |
| | CYP4C51 | AAEL008018 | CAATCGACAAGCTCAGACCA | GCATTATGCGTCCGATTTCT |
| | CYP4C52 | AAEL008023 | TTCCTCGATCGGGTCATTAG | GCTTTTCTCCACCAGCTTTG |
| | CYP4D23 | AAEL007816 | GTTCAACAAGCGCAAGATCA | TTTATCGGAGTTCCCATTGC |
| | CYP4D24 | AAEL007815 | TACTTCACCCCGTACCGAAG | AGGGGTCTCCCGTCTACTGT |
| | CYP4D37 | AAEL007795 | GGAGACGGTTTGCTTCTGAG | CAAAGCGTACAGCAGCACAT |
| | CYP4D38 | AAEL007807 | CAAGCAACCCGATGAATTTT | CGAGCCAGTTGGAGAGGTAG |
| | CYP4D39 | AAEL007808 | CGTCGCAGCAATAAAACTCA | CCTCTCGATGGTGAGGAAAA |
| | CYP4G35 | AAEL008345 | GGACCGATGGCTTCAGAATA | GCATCAAGCAAAAGCAACAA |
| | CYP4G35 | AAEL006824 | GGTCGTCAAGCAGAAGAAGG | GAAATCCAGATCGTCCCTGA |
| | CYP4G36 | AAEL004054 | AACACCAATAGCGTGGAAGG | CCCATCATCGAAAGGAAGAA |
| | CYP4H28 | AAEL003380 | ACACCGAAGGTGAAACCAAG | GGGCCATCAACGAGAAGTAA |
| | CYP4H29 | AAEL007830 | TGCAGGCTGTCAAAGAAATG | GATTCTTGCCTTGCTTGCTC |
| | CYP4H30 | AAEL003399 | GCTGCTGAAAATAGCGAACC | GTCCCCCAGGAATAGGACAT |
| | CYP4H31 | AAEL002085 | ACAATTCGTGACGGTGTTCA | TTGGATTCTTCTGGGCATTC |
| | CYP4H32 | AAEL007812 | ATCCAAATGCTTGGGAACAG | CTTCCTCTCGGATGTCTTCG |
| | CYP4H33 | AAEL013798 | CAGTAATTGATGCGCGAAGA | ATGACCCTCGAACATGAAGG |
| | CYP4J13 | AAEL013555 | CAGGACCGTTGGAAGTTGAT | AGAGCGCACAGTACCGTTTT |
| | CYP4J14 | AAEL013554 | AAGTTGTCCACCGTTTCTGG | GAACGAATGAACCGGAAGAA |
| | CYP4J15v1 | AAEL013556 | GCTGGATTCGCTTTTACTCG | GAAAGCTCGGGATGACTGAG |
| | CYP4J15v2 | AAEL014829 | AAACATCGATGGGCGTTAAG | AGCCGCTTTATAGCCTGTCA |
| | CYP4J16 | AAEL015663 | AACGGATCATGAACCCTCTG | TTCCGCCAGCAGAAGACTAT |
| | CYP4J17 | AAEL015370 | AGAATTCCCCTACCGCTTGT | GGCCAGTAAGGTATCCAGCA |
| | CYP4J17 | AAEL014019 | GGAGGAGATCGAAACCATGA | ACTGTGCATCCTCCAAAACC |
| | CYP4K3 | AAEL007798 | GGAAGTGCAACGGAAATTGT | CTTCGGACTTTGAGGCGTAG |
| | CYP4AR2 | AAEL010154 | CGGAGGTTTGCAATGATTCT | CGTCTGGGTACTTTGCCATT |
| | CYP325E3 | AAEL000338 | CAATAGGGTGTTTCGGGAGA | CTGTTAAGGATGCGGGTGTT |
| | CYP325G2 | AAEL012766 | CTTATCGGTTGTGGCCATCT | CTGCATATCTTCCGGGTTGT |
| | CYP325G3 | AAEL012772 | TACCCTTTGATCGGAAGTGC | ACCGGTGAAGTGAACAGTCC |
| | CYP325K2 | AAEL005771 | ATTTTGCCCGCTATCTTCCT | TTCTCCTGGCAATAGGGATG |
| | CYP325K3 | AAEL005788 | ATTCCGAAGGGAACTGTCCT | CAAGTCGGTGCTGAGTTCAA |
| | CYP325L1v1 | AAEL011770 | TCGGTGGAAACGAAACTACC | GTTCGTCCGAGGATTGTTGT |
| | CYP325M1 | AAEL012773 | AGACGAAAAGTTCGCAGCAT | CCTCTTGATAGCAGCGTTCC |
| | CYP325M2 | AAEL012769 | TTCGTTCTTTTGGGTTCCAC | TGCTGCTTTCCAACGTATTG |
| | CYP325M2 | AAEL015591 | TTCGTTCTTTTGGGTTCCAC | TGCTGCTTTCCAACGTATTG |
| | CYP325M3 | AAEL012765 | CGATCTGTGTGGAGAGCAAA | GTTTTCGCCCGTTGTTTCATT |
| | CYP325M4 | AAEL011769 | CGTTGAATCCTTCGTTTGGT | GGCCGTTGCATAGATTTGAT |
| | CYP325M5 | AAEL011761 | TGGAAAAATCAACGGAAAGC | TCGCTGCATATCGAAGTCAC |
| | CYP325N1 | AAEL012770 | GTACCTTGAAGCGCAAGAGG | TGTTCAGCATTCCTTGCTTG |
| | CYP325N2 | AAEL012762 | CTTGCCCGGATAGAAATCAA | TCCGGGTTGAAGTTTTTGAC |
| | CYP325P1 | AAEL000340 | CGTGGTTGATTTCGGAGTTT | GCCTGGGTGTGATTTCTGTT |
| | CYP325Q1 | AAEL006044 | ACCACGGAAGCTCTGAAAAA | CATCTGGTCCCCATACATCC |
| | CYP325Q2 | AAEL015563 | ACGAAAGTGCGGAAAGAAGA | CAGGTGTAGGAAACGGCATT |
| | CYP325R1 | AAEL005775 | CGGCTTACTCATGGGTTGTT | TATCCACTGGAGTCCCTTCG |
| | CYP325S1 | AAEL000326 | CCGATTTTCTTCGACAGCTC | GCAAAATTCTCCGGATCAAA |
| | CYP325S2 | AAEL000325 | GCCGAAATCATGGAACACTT | CCACATATCCGCTCTTCGAT |
| | CYP325S3 | AAEL000357 | TTGCTCGGCAGTGTATCAAG | TCCGGTAGGAAATTGTCTGG |
| | CYP325T2 | AAEL012761 | GAGTTTGCCATCCGGATCTA | CCTGCTGCAACACTTTTCAA |
| | CYP325T2 | AAEL015475 | CTCATGGCCTATGCCTGTTT | GCCATGTTTTGCCTTACGAT |
| | CYP325U1 | AAEL000320 | GGGGAAAAATGGTGAGGAAT | ATTCAGTGCCTTACGCTGCT |
| | CYP325X1 | AAEL005695 | CTGTACCGGTTTGCTGGATT | TCGTCATCTTCTCGCAACAC |

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**Table 1. Continued**

| Primer | Gene[a] | Vectorbase[b] | Forward primer (5′–3′) | Reverse primer (5′–3′) |
|---|---|---|---|---|
| | CYP325X2 | AAEL005696 | TGTCGTTCTACCCGGAAATC | TCGAACTCGGCCATATTTTC |
| | CYP325X4 | AAEL005700 | GGCTCAACTCCAGCTTCAAC | CGAATTCCTCTTTCCCTTCC |
| | CYP325Y1 | AAEL006257 | GAAATCGTGCTCGATGGAAT | AGATAGGCAAATGGGTGACG |
| | CYP325Y2v2 | AAEL015362 | AGGAAGCCCTCCGTATTTGT | ATGCCTTTGCTGAACTGCTT |
| | CYP325Y3 | AAEL006246 | GGCATACGCATCCCTAAAGA | TCTGCATAATCCGCAACAAA |
| | CYP325Y3 | AAEL015361 | CAATCGCTTGGTGAGGATTT | CCTCCGGGTACATTGCTAGA |
| | CYP325Z1 | AAEL010273 | CACCAAATCCAAGCCAAAGT | GTCTTTCCGCCTGTGAAGAG |
| | CYP325AA1 | AAEL004012 | TGCTTTCGTGGATCGTAGTG | CACCAGCTCTGGATGCTGTA |
| | CYP12F5 | AAEL001960 | ACAAGGAGAAAGCTGGCAAA | CATCGAGAACTCCCAATCGT |
| | CYP12F6 | AAEL002005 | TACATCGTTGACTCCGGACA | CGAAGCGATCACTTTGTTGA |
| | CYP12F7 | AAEL002031 | CTGGAAACGATGGGTGTTCT | GATAACCGCGTCATCAACCT |
| | CYP12F8 | AAEL006827 | TGGATAAGGTTGCCCTTCAG | TCTCCAGATCGAGGGAAGAA |
| | CYP49A1 | AAEL008638 | GTGCATCAAAGAAACGCTGA | CGGTCTGGTTCTGGGAAATA |
| | CYP301A1 | AAEL014594 | CCTGGAACCGAACTTGACAT | CGTCCTTCACCCAAGTTCAT |
| | CYP302A1v1 | AAEL011463 | TTTCGATGTACGGTTGGACA | GCTTTCGATACGCTGGAGTC |
| | CYP302A1v2 | AAEL015655 | TTTCGATGTACGGTTGGACA | GCTTTCGATACGCTGGAGTC |
| | CYP314A1 | AAEL010946 | GCGGAGACAAGCAAAAGAAC | ACGATTTCGGCGATTGTATC |
| | CYP315A1 | AAEL011850 | ATTCATTGGACGCTTTTTGG | TCCCTTCGTAACCACCTTTG |
| | P450 reductase | AAEL003349 | TTCCTTCCCCGCTTTTATCT | CTGTGTAGCGGTGCTTGTGT |
| Drosophila constructs | CYP4D24 | AAEL007815 | CCGCTCGAGCAAAATGCTTATCT TATTGGCT | CTAGCTCGAGCCGCACCCTGCT TCTGATCCT |
| | CYP4H29 | AAEL007830 | CCGGAATTCCAAAATGGTGCCT CTTCTGATG | CTAGCTCGAGTCGTGGCACAAT CTTCACAAA |
| | CYP4J15v1 | AAEL013556 | CCGGAATTCCAAAATGTTGCTT ATTCTAACGC | CTAGCTCGAGTCTCCTCTCAA ACCTAACCTC |
| | CYP4H33 | AAEL013798 | CCGGAATTCCAAAATGGATTTC CTAACGAAT | CTAGCTCGAGAATTCTTTCCA CTAGCTTAAT |

[a] Nomenclature for the cytochrome P450 genes was taken for the *Ae. aegypti* cytochrome P450 database at: http://drnelson.uthsc.edu/CytochromeP450.html.

[b] Vectorbase *Ae. aegypti* predicted gene set vs. AaegL1.1. (http://aaegypti.vectorbase.org/).

*aegypti* (Strode et al. 2008, Pridgeon et al. 2009, Poupardin et al. 2010, Fonseca-Gonzalez et al. 2011, Bariami et al. 2012, Saavedra-Rodriguez et al. 2012, Strode et al. 2012) and other mosquitoes, including *Anopheles gambiae* Giles (Boonseupsekul et al. 2008; McLaughlin et al. 2008; Müller et al. 2008; Stevenson et al. 2011, 2012), have been extensively studied. Collectively, these studies suggest that cytochrome P450s-mediated detoxification play an important role the resistance of *Ae. aegypti* to pyrethroid insecticides. Multiple studies have investigated the functional role of insect cytochrome P450s (Joussen et al. 2008; Müller et al. 2008; Zhu et al. 2010; Stevenson et al. 2011, 2012; Yang and Liu 2011; Chandor-Proust et al. 2013), especially on cytochrome P450s in families CYP6 and CYP9. However, information on the functional role of family 4 cytochrome P450s in pyrethroid resistance is scarce.

In Puerto Rico, mosquito control has relied heavily on the usage of pyrethroid insecticide permethrin. However, higher and higher concentrations were needed for successful mosquito control. To understand whether any field strain of *Ae. aegypti* in Puerto Rico has developed resistance to permethrin, a field strain was randomly collected from San Juan, Puerto Rico, in October 2008. The objectives of this study were to: 1) determine whether the Puerto Rico strain of *Ae. aegypti* was resistance to permethrin, 2) determine whether cytochrome P450-mediated detoxification was involved in the resistance in this Puerto Rico strain if the strain was resistant to permethrin, 3) identify up-regulated P450 genes in the Puerto Rico strain of *Ae. aegypti* if the strain was resistant to permethrin, and 4) determine whether any of the up-regulated family 4 cytochrome P450 gene could confer resistance in *Drosophila* to permethrin through transgenic work.

## Materials and Methods

**Mosquito Strains.** The Orlando strain of *Ae. aegypti* has been continuously reared at the Center for Medical, Agricultural, and Veterinary Entomology (CMAVE), U.S. Department of Agriculture–Agricultural Research Service (USDA–ARS) in Gainesville, FL, since 1952 (Allan 2011, Clark et al. 2011). The Puerto Rico strain of *Ae. aegypti* was collected in urban San Juan, Puerto Rico in October 2008. Both mosquito strains were reared in the Insectary of the Mosquito and Fly Research Unit at CMAVE, USDA–ARS. Female adults were used for all experiments because only females take bloodmeals and are of concern to the general public. Eggs were hatched by placing a square of a paper towel with eggs in a flask filled with 1,000 ml of distilled water containing 40 mg of larval diet (3:2 brewer's yeast : liver powder [MP Biomedicals, Irvine, CA]). The hatched larvae were held overnight in the flask and 200 larvae were transferred to a 4-liter plastic tray containing 2 liters of distilled water. Larval diet was added to each tray according to the following schedule: Day 1, 80 mg; D 3, 40 mg; D 4, 80 mg; D 5, 120 mg; and D 6, 150 mg. Mosquitoes were reared in an environmental chamber set with a temperature profile representing a simulated summer day regime (ranging from 22 to 30°C) and 80% relative

**Table 2. Resistance ratio of the Puerto Rico *Ae. aegypti* strain compared with the Orlando strain in the presence or absence of the P450 inhibitor PBO**

| Strain | $LD_{50}$ (95% CI) μg per insect | Slope (SE) | $\chi^2$ | df | Fold resistance |
|---|---|---|---|---|---|
| Orlando | | | | | |
| −PBO | 2.08 by $10^{-4}$ (1.18 by $10^{-4}$ − 4.11 by $10^{-4}$) | 2.89 (0.44) | 8.02 | 4 | 1.00 |
| +PBO | 1.42 by $10^{-4}$ (8.80 by $10^{-5}$ − 2.26 by $10^{-4}$) | 2.08 (0.29) | 6.37 | 5 | 0.68 |
| Puerto Rico | | | | | |
| −PBO | 1.52 by $10^{-2}$ (9.86 by $10^{-3}$ − 2.99 by $10^{-2}$) | 1.48 (0.26) | 1.39 | 7 | 73.07 |
| +PBO | 3.28 by $10^{-3}$ (2.02 by $10^{-3}$ − 5.85 by $10^{-3}$) | 0.96 (0.14) | 3.99 | 7 | 15.76 |

humidity. Incandescent lights were set to a crepuscular profile with a photoperiod of 14:10 (L:D) h, including 2 h of simulated dawn and 2 h of simulated dusk. Adults were held in a screened cage and provided 10% sucrose ad libitum.

**Topical Application Bioassays.** Topical application bioassays were performed using published procedures (Pridgeon et al. 2007). Briefly, 2- to 5-d-old adults were collected by gentle aspiration, anesthetized at 4°C for 60 min, then females were sorted from males and three 250-cc plastic cups containing 10 adult females each were covered with two layers of tulle mesh and provided with cotton balls saturated with 10% sucrose for feeding. In total, three cups (10 insect per cup) were used for each permethrin dose and all experiments were repeated in triplicate. The $LD_{50}$ values were determined using six concentrations that resulted in mortality ranging from 10 to 90% along with an acetone control and untreated controls. Before application, females were anesthetized for 30 s with $CO_2$ and placed on a 4°C chill table (BioQuip Products, Rancho Dominguez, CA). A 0.5-μl droplet of either acetone (controls), or permethrin dissolved in acetone was applied directly to the dorsal surface of the thorax using a 700 series syringe (Hamilton, Reno, NV). To determine whether cytochrome P450s were involved in the resistance, piperonyl butoxide (PBO, the inhibitor of P450s) was applied topically to adult female *Ae. aegypti* 1 h before the application of the permethin to allow for the PBO to inhibit the cytochrome P450 activity in the mosquitoes. The dose of 0.4 μg per mosquito was used for bioassays in the presence of a PBO inhibitor because it was the dose that resulted in no mortality in Orlando or Puerto Rico strains of *Ae. aegypti.*

**RNA Extraction, cDNA Synthesis, Primer Design, and Quantitative Polymerase Chain Reaction (qPCR).** Total RNA was isolated from Orlando strain for Puerto Rico strain of *Ae. aegypti* using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. First strand cDNA synthesis was conducted on 5 μg of total RNA in a 20 μl reaction mixture using oligo-$dT_{20}$ primer (Invitrogen, Carlsbad, CA). The resulting cDNA was further diluted fivefold as described previously (Pridgeon et al. 2009). qPCR was performed using the SYBR Green PCR Master Mix on an ABI 7300 quantitative PCR System (Applied Biosystems, Foster City, CA). The template used to design primers (Table 1) was based on the P450 sequences of the Liverpool strain of *Ae. aegypti* (GenBank accession no. CH478182). For all cDNA samples, *Ae. aegypti* actin (GenBank accession no. DQ440059) primers were included as an internal control to normalize the variation of cDNA amount as described previously (Pridgeon et al. 2009). Primers used for the amplification of the actin gene were Actin-152F (5′-AGGACTCGTACGTCGGTGAC-3′) and Actin-590R (5′-CGTTCAGTCAGGATCTTC-3′). The qPCR thermal cycling parameters were 50°C for 2 min, 95°C for 10 min, followed by 40 cycle of 95°C for 15 s and 60°C for 1 min. All qPCR was replicated three times. The relative expression level of each of the cytochrome P450 genes was normalized to actin within mosquito strain and then the fold change in gene expression level in Puerto Rico strain compared with that in Orlando strain was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Statistical analysis was performed using a Welch's *t*-test in R (R Core Team 2013).

**Functional Analysis of Four Selected Cytochrome P450 Genes.** Four up-regulated family 4 cytochrome P450 genes, *CYP4D24*, *CYP4H29*, *CYP4J15v1*, and *CYP4H33*, were used in this study. The full lengths of the four up-regulated P450 genes from the Puerto Rico strain of *Ae. aegypti* were amplified from cDNA using platinum *Taq*DNA polymerase High Fidelity (Invitrogen, Carlsbad, CA) with gene-specific primers (Table 1) based on the 5′ and 3′ end sequences of the genes. PCR products of the full length genes were purified using a QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The purified PCR products were ligated into pCR 2.1 vector using the Original TA Cloning kit (Invitrogen, Carlsbad, CA) as described by the manufacturer. The full lengths of the genes were cloned in One Shot TOPO 10F′ cells using the One Shot TOP10F′ Chemically Competent *E. coli* kit (Invitrogen, Carlsbad, CA). Cloning and sequence analyses of the cDNAs were repeated at least three times and three TA clones from each replication were verified by sequencing. The clones were then subcloned into the pUASTattB vector (a gift from Dr. Johannes Bischof, University of Zurich; Brand and Perrimon 1993, Bischof et al. 2007). The plasmid of each pUASTattB-up-regulated gene was transformed into the germ line of *Drosophila melanogaster* Meigen (Bloomington stock #24484, genotype M{vas-int.Dm}ZH-2A, M{3xP3-RFP.attP′}ZH-58A), resulting in site-specific integration on chromosome 2R (Bateman et al. 2006; Rainbow Transgenic Flies Inc., Camarillo, CA). Flies were then reciprocally crossed against a $W^{1118}$ strain to obtain transgenic *D. melanogaster* with the orange eye phenotype. The flies were then balanced against a *D. melanogaster* balancer

**Table 3. Relative cytochrome P450 gene expression values in the pyrethroid-resistant Puerto Rico strain compared with the pyrethroid-susceptible Orlando strain**

| P450[a] | AAEL[b] gene no. | Fold up-regulated in Puerto Rico compared with Orlando |
|---|---|---|
| CYP15B1 | AAEL002067 | 2.51 ± 0.15[†] |
| CYP18A1 | AAEL004870 | 1.29 ± 0.21 |
| CYP303A1ae | AAEL012144 | 1.15 ± 0.16 |
| CYP304B2xx/yy | AAEL014412 | 1.29 ± 0.17 |
| CYP304B3yy/xx | AAEL014411 | 1.00 ± 0.23 |
| CYP304C1 | AAEL014413 | 1.33 ± 0.17 |
| CYP305A6 | AAEL002071 | 1.41 ± 0.26 |
| CYP305A5 | AAEL002043 | 1.38 ± 0.39 |
| CYP306A1 | AAEL004888 | 0.86 ± 0.33 |
| CYP307A1 | AAEL009762 | 1.30 ± 0.28 |
| CYP307A1 | AAEL009768 | 1.48 ± 0.28 |
| CYP307B1 | AAEL006875 | 1.29 ± 0.17 |
| CYP6F2 | AAEL014678 | 4.38 ± 1.38[†] |
| CYP6F3 | AAEL014684 | 1.92 ± 0.62 |
| CYP6M5 | AAEL009117 | 0.80 ± 0.38 |
| CYP6M6 | AAEL009128 | 0.69 ± 0.21 |
| CYP6M10 | AAEL009125 | 0.99 ± 0.35 |
| CYP6M11 | AAEL009127 | 5.70 ± 0.44[†] |
| CYP6N6 | AAEL009126 | 1.62 ± 0.16 |
| CYP6N9 | AAEL009121 | 2.31 ± 0.17[†] |
| CYP6N11 | AAEL009119 | 0.33 ± 0.01[‡] |
| CYP6N11 | AAEL009138 | 1.50 ± 0.23 |
| CYP6N12 | AAEL009124 | 3.38 ± 0.24[†] |
| CYP6N13 | AAEL009137 | 3.53 ± 0.31[†] |
| CYP6N14 | AAEL009133 | 1.45 ± 0.12 |
| CYP6N15 | AAEL009122 | 1.61 ± 0.17 |
| CYP6N16 | AAEL010151 | 1.42 ± 0.13 |
| CYP6N17 | AAEL010158 | 1.48 ± 0.25 |
| CYP6P12v1 | AAEL012491 | 1.46 ± 0.13 |
| CYP6S3 | AAEL009120 | 1.7 ± 0.09 |
| CYP6Y3 | AAEL009132 | 1.5 ± 0.10 |
| CYP6Z6 | AAEL009123 | 3.16 ± 0.07[†] |
| CYP6Z7 | AAEL009130 | 0.89 ± 0.70 |
| CYP6Z8 | AAEL009131 | 0.06 ± 0.01[‡] |
| CYP6Z9 | AAEL009129 | 1.31 ± 0.06 |
| CYP6AA5v1 | AAEL012492 | 1.61 ± 0.09 |
| CYP6AG3 | AAEL007024 | 2.33 ± 0.06[†] |
| CYP6AG5 | AAEL006984 | 1.18 ± 0.14 |
| CYP6AG6 | AAEL006992 | 1.29 ± 0.16 |
| CYP6AG7 | AAEL006989 | 2.28 ± 0.16[†] |
| CYP6AG8 | AAEL003890 | 1.37 ± 0.19 |
| CYP6AH1 | AAEL007473 | 1.51 ± 0.11 |
| CYP6AH1 | AAEL015641 | 1.74 ± 0.26 |
| CYP6AK1 | AAEL004941 | 1.92 ± 0.19 |
| CYP6AL1 | AAEL008889 | 2.06 ± 0.11 |
| CYP6AL3 | AAEL009656 | 0.83 ± 0.16 |
| CYP6BB2 | AAEL014893 | 3.01 ± 0.25[†] |
| CYP6BZ1 | AAEL012494 | 1.67 ± 0.15 |
| CYP6CA1 | AAEL014680 | 2.15 ± 0.64 |
| CYP6CB1 | AAEL002046 | 14.46 ± 0.05[†] |
| CYP6CB1 | AAEL009018 | 10.60 ± 0.13[†] |
| CYP6CB2 | AAEL002872 | 0.52 ± 0.32 |
| CYP6CC1v1 | AAEL014890 | n/d |
| CYP6CD1 | AAEL005006 | 1.52 ± 0.23 |
| CYP9J2 | AAEL006805 | 12.17 ± 1.23[†] |
| CYP9J6 | AAEL002638 | 1.86 ± 0.18 |
| CYP9J7 | AAEL014606 | 1.72 ± 0.17 |
| CYP9J8 | AAEL006811 | 2.11 ± 0.82 |
| CYP9J9 | AAEL006793 | 2.45 ± 0.39 |
| CYP9J9 | AAEL014605 | 2.83 ± 0.26 |
| CYP9J10 | AAEL006798 | 2.89 ± 0.28[†] |
| CYP9J15 | AAEL006795 | 0.70 ± 0.53 |
| CYP9J16 | AAEL006815 | 0.88 ± 0.39 |
| CYP9J17 | AAEL009699 | 0.78 ± 0.24 |
| CYP9J17 | AAEL006784 | 0.31 ± 0.06[‡] |
| CYP9J18v1 | AAEL006804 | 0.77 ± 0.41 |
| CYP9J19 | AAEL006810 | 1.41 ± 0.20 |
| CYP9J19 | AAEL014611 | 0.04 ± 0.01[‡] |
| CYP9J20v1 | AAEL006814 | 3.29 ± 0.44[†] |
| CYP9J20v2 | AAEL014604 | 2.04 ± 0.50 |
| CYP9J21 | AAEL014612 | 3.94 ± 0.43[†] |
| CYP9J22 | AAEL006802 | 2.30 ± 0.32 |
| CYP9J23 | AAEL014615 | 3.77 ± 0.31[†] |
| CYP9J24 | AAEL014613 | 1.84 ± 0.35 |
| CYP9J26 | AAEL014609 | 2.76 ± 0.24[†] |
| CYP9J27 | AAEL014616 | 2.85 ± 0.16[†] |
| CYP9J27 | AAEL014607 | 3.17 ± 0.19[†] |
| CYP9J28 | AAEL014617 | 1.36 ± 0.3 |
| CYP9J29 | AAEL014610 | 0.39 ± 0.07[‡] |
| CYP9J30 | AAEL014603 | 1.37 ± 0.33 |
| CYP9J31 | AAEL002633 | 2.30 ± 0.14[†] |
| CYP9J32 | AAEL008846 | 1.16 ± 0.15 |
| CYP9M4 | AAEL001320 | 1.02 ± 0.30 |
| CYP9M5 | AAEL001288 | 1.18 ± 0.34 |
| CYP9M6 | AAEL001312 | 1.83 ± 0.23 |
| CYP9M7 | AAEL001292 | 1.73 ± 0.38 |
| CYP9M8 | AAEL009591 | 1.55 ± 0.21 |
| CYP9M9 | AAEL001807 | 1.01 ± 0.14 |
| CYP9AE1 | AAEL003748 | 0.38 ± 0.08[‡] |
| CYP329B1 | AAEL003763 | 1.51 ± 0.13 |
| CYP4C38 | AAEL012266 | 1.09 ± 0.15 |
| CYP4C50 | AAEL008017 | 1.20 ± 0.19 |
| CYP4C51 | AAEL008018 | 0.85 ± 0.36 |
| CYP4C52 | AAEL008023 | 0.61 ± 0.42 |
| CYP4D23 | AAEL007816 | 1.68 ± 0.17 |
| CYP4D24 | AAEL007815 | 2.81 ± 0.20[†] |
| CYP4D37 | AAEL007795 | 1.47 ± 0.30 |
| CYP4D38 | AAEL007807 | 1.44 ± 0.10 |
| CYP4D39 | AAEL007808 | 1.54 ± 0.07 |
| CYP4G35 | AAEL008345 | 3.03 ± 0.07[†] |
| CYP4G35 | AAEL006824 | 3.44 ± 0.20[†] |
| CYP4G36 | AAEL004054 | 2.84 ± 0.16[†] |
| CYP4H28 | AAEL003380 | 1.27 ± 0.71 |
| CYP4H29 | AAEL007830 | 2.52 ± 0.43[†] |
| CYP4H30 | AAEL003399 | 3.85 ± 0.50[†] |
| CYP4H31 | AAEL002085 | 1.36 ± 0.51 |
| CYP4H32 | AAEL007812 | 0.36 ± 0.02[‡] |
| CYP4H33 | AAEL013798 | 2.73 ± 0.13[†] |
| CYP4J13 | AAEL013555 | 1.05 ± 0.15 |
| CYP4J14 | AAEL013554 | 1.00 ± 0.11 |
| CYP4J15v1 | AAEL013556 | 2.30 ± 0.18[†] |
| CYP4J15v2 | AAEL014829 | 3.85 ± 0.46[†] |
| CYP4J16 | AAEL015663 | 1.06 ± 0.29 |
| CYP4J17 | AAEL015370 | 1.23 ± 0.42 |
| CYP4J17 | AAEL014019 | 0.44 ± 0.03[‡] |
| CYP4K3 | AAEL007798 | 1.19 ± 0.28 |
| CYP4AR2 | AAEL010154 | 1.09 ± 0.23 |
| CYP325E3 | AAEL000338 | 0.76 ± 0.64 |
| CYP325G2 | AAEL012766 | 2.06 ± 0.31 |
| CYP325G3 | AAEL012772 | 3.61 ± 1.20[†] |
| CYP325K2 | AAEL005771 | 1.23 ± 0.24 |
| CYP325K3 | AAEL005788 | 2.08 ± 0.19 |
| CYP325L1v1 | AAEL011770 | 1.66 ± 0.07 |
| CYP325M1 | AAEL012773 | 1.28 ± 0.18 |
| CYP325M2 | AAEL012769 | 1.41 ± 0.19 |
| CYP325M2 | AAEL015591 | 1.54 ± 0.19 |
| CYP325M3 | AAEL012765 | 0.31 ± 0.02[‡] |
| CYP325M4 | AAEL011769 | 2.72 ± 0.28[†] |
| CYP325M5 | AAEL011761 | 1.40 ± 0.18 |
| CYP325N1 | AAEL012770 | 0.76 ± 0.34 |
| CYP325N2 | AAEL012762 | 0.98 ± 0.14 |
| CYP325P1 | AAEL000340 | 1.66 ± 0.13 |
| CYP325Q1 | AAEL006044 | 1.16 ± 0.13 |
| CYP325Q2 | AAEL015563 | 1.44 ± 0.30 |
| CYP325R1 | AAEL005775 | 1.76 ± 0.09 |
| CYP325S1 | AAEL000326 | 0.51 ± 0.41 |
| CYP325S2 | AAEL000325 | 0.68 ± 0.10 |
| CYP325S3 | AAEL000357 | 0.56 ± 0.05 |
| CYP325T2 | AAEL012761 | 1.02 ± 0.22 |
| CYP325T2 | AAEL015475 | 0.81 ± 0.13 |

Continued on following page

**Table 3. Continued**

| P450[a] | AAEL[b] gene no. | Fold up-regulated in Puerto Rico compared with Orlando |
|---|---|---|
| CYP325U1 | AAEL000320 | 0.14 ± 0.01 |
| CYP325X1 | AAEL005695 | 1.19 ± 0.13 |
| CYP325X2 | AAEL005696 | 1.37 ± 0.12 |
| CYP325X4 | AAEL005700 | 1.39 ± 0.14 |
| CYP325Y1 | AAEL006257 | 1.09 ± 0.20 |
| CYP325Y2v2 | AAEL015362 | 0.50 ± 0.23 |
| CYP325Y3 | AAEL006246 | 1.21 ± 0.20 |
| CYP325Y3 | AAEL015361 | 1.31 ± 0.22 |
| CYP325Z1 | AAEL010273 | 1.30 ± 0.13 |
| CYP325AA1 | AAEL004012 | 1.51 ± 0.21 |
| CYP12F5 | AAEL001960 | 0.47 ± 0.27 |
| CYP12F6 | AAEL002005 | 2.14 ± 0.19* |
| CYP12F7 | AAEL002031 | 1.37 ± 0.11 |
| CYP12F8 | AAEL006827 | 2.30 ± 0.29† |
| CYP49A1 | AAEL008638 | 1.45 ± 0.14 |
| CYP301A1 | AAEL014594 | 1.84 ± 0.15 |
| CYP302A1v1 | AAEL011463 | 1.91 ± 0.15 |
| CYP302A1v2 | AAEL015655 | 1.46 ± 0.22 |
| CYP314A1 | AAEL010946 | 0.77 ± 0.16 |
| CYP315A1 | AAEL011850 | 1.92 ± 0.07 |
| NADPH P450 reductase | AAEL003349 | 4.76 ± 0.18† |

[a] Nomenclature for the cytochrome P450 genes was taken for the *Ae. aegypti* cytochrome P450 database at: http://drnelson.uthsc.edu/CytochromeP450.html.

[b] Vectorbase *Ae. aegypti* predicted gene set vs. AaegL1.1. http://aaegypti.vectorbase.org/.

* Significantly up-regulated (more than twofold) at the $P < 0.05$ level of significance.

† Significantly up-regulated (more than twofold) at the $P < 0.01$ level of significance.

‡ Significantly down-regulated (more than twofold) at the $P < 0.01$ level of significance.

strain (Bloomington stock #6312, genotype: w[1118]/Dp(1;Y)y[+]; sna[Sco]/CyO, P{ry[+t7.2] = sevRas1.V12}FK1) to generate a homozygous line containing the cytochrome P450 transgene on chromosome 2R. The insertion of the up-regulated genes in the transgenic fruit fly lines were further confirmed using reverse transcription-polymerase chain reaction (RT-PCR). Transgenic virgin female *D. melanogaster* were then crossed with male GAL4-expressing *D. melanogaster* (Bloomington stock #3954, genotype: P{Act5C-GAL4}17bFO1), which expresses GAL4 under control of the Act5C promoter, resulting in ubiquitous nontissue-specific expression. The F1 generation of these crosses expressed GAL4 and contained a single copy of the cytochrome P450 transgene, which was under control of the upstream activating sequence (UAS) enhancer. Permethrin toxicity bioassays were then conducted on 2–3 d posteclosion female *Drosophila* of F1 UAS–GAL4 crosses to examine the toxicity of pyrethroids to transgenic flies. Briefly, serial concentrations of each pyrethroid solution in acetone, ranging from 25 to 100 ng/μl that gave >0 and <100% mortality to the tested insects were prepared. Two hundred microliter of each permethrin concentration solution was evenly coated on the inside of individual 20-ml glass scintillation vials. Then, 15 female flies were transferred to each of the prepared vials, and three vials were used for each concentration for each bioassay replicate. The vials were plugged with cotton balls soaked with 5% sucrose and the mortality was scored after a 24-h exposure to the pyrethroids. Each bioassay was independently replicated three times using only flies that exhibited the correct morphological marker (GAL4 red eyes). The *D. melanogaster* strain (Bloomington stock #24484, genotype: M{vas-int.Dm}ZH-2A, M{3xP3-RFP.attP'}ZH-58A) containing the empty pUAST vector donated insert, but no transgene was used as the control reference strain according to the identical crossing protocol of virgin control females with GAL4 expressing males to obtain the F1 generation for testing. Preliminary testing determined that vials coated with 2 μg of permethrin resulted in nearly complete mortality of the empty-vector control line. Subsequently, the lowest insecticide concentration at 5 μg of permethrin resulted in 100% mortality of the control mosquitoes for all bioassay replicates. Therefore, the concentration of 5 and 10 μg per vial of permethrin were used to test the transgenic flies' susceptibility to permethrin. All tests were run at 27°C and mortality was assessed at 24 h postexposure. All *D. melanogaster* were reared on Jazz-Mix *Drosophila* food (Fisher, KS City, MO) at 25 ± 2°C under a photoperiod of 12:12 (L:D) h following standard protocols (Ashburner et al. 2005).

## Results and Discussion

Results of the topical application bioassays are summarized in Table 2. The $LD_{50}$ value of permethrin to Orlando strain of *Ae. aegypti* was 2.08 by $10^{-4}$ μg per mosquito, whereas that of permethrin to the Puerto Rico strain of *Ae. aegypti* was 1.52 by $10^{-2}$ μg per mosquito (Table 2). Therefore, the Puerto Rico strain of *Ae. aegypti* was 73-fold resistant to permethrin compared with that of the Orlando strain (Table 2). When PBO (the inhibitor of cytochrome P450) was present, the resistance of the Puerto Rico strain to permethrin was decreased to 15-fold (Table 2). These results suggested that cytochrome P450-mediated detoxification might play a role in the resistance of the Puerto Rico strain of *Ae. aegypti* to permethrin.

Results of transcriptional levels of P450 genes determined by qPCR were summarized in Table 3. Of the 164 cytochrome P450s selected for this study, 33 were significantly ($P < 0.05$) up-regulated more than twofold (Table 3) and eight were significantly ($P < 0.05$) down-regulated more than twofold (Table 3). For the remaining 123 cytochrome P450 genes, no significant difference ($P > 0.05$) was observed between the transcriptional level in the Puerto Rico strain and that of the Orlando strain (Table 3). Although the Puerto Rico field population is geographically distinct from the Orlando strain, the transcriptional levels of the majority (123 of 164) of the P450s in the two strains were not significant different, suggesting that not all P450s play roles in resistance to permethrin. Our results also further suggest that resistance is developed under selection pressure, as permethrin was widely used in Puerto Rico to control mosquitoes.

Previous studies by multiple researchers have discovered the up-regulation of cytochrome P450 genes in insecticide-resistant *Ae. aegypti* (Strode et al. 2008, Marcombe et al. 2009, Bariami et al. 2012, Saavedra-Rodri-

**Table 4. Transcriptional level of differentially expressed cytochrome in the Puerto Rico strain compared with that in the Orlando strain of *Ae. aegypti***

| Gene[b] | Name[a] | Fold | Reported up-regulation in insecticide-resistant *Ae. aegypti* |
|---|---|---|---|
| AAEL002067 | *CYP15B1* | 2.51 ± 0.03† | |
| AAEL007024 | *CYP6AG3* | 2.33 ± 0.05† | |
| AAEL006989 | *CYP6AG7* | 2.28 ± 0.08† | |
| AAEL014893 | *CYP6BB2* | 3.01 ± 0.18† | (Bariami et al. 2012, Saavedra-Rodriguez et al. 2012) |
| AAEL002046 | *CYP6CB1* | 14.46 ± 0.28† | (Strode et al. 2008, Bariami et al. 2012, Saavedra-Rodriguez et al. 2012) |
| AAEL009018 | *CYP6CB1* | 10.60 ± 0.41† | (Strode et al. 2008, Bariami et al. 2012) |
| AAEL014678 | *CYP6F2* | 4.38 ± 0.11† | |
| AAEL009127 | *CYP6M11* | 5.70 ± 0.31† | (Poupardin et al. 2008, Marcombe et al. 2009, Bariami et al. 2012) |
| AAEL009121 | *CYP6N9* | 2.31 ± 0.04† | (Bariami et al. 2012) |
| AAEL009124 | *CYP6N12* | 3.38 ± 0.16† | (Bariami et al. 2012) |
| AAEL009137 | *CYP6N13* | 3.53 ± 0.01† | |
| AAEL009123 | *CYP6Z6* | 3.16 ± 0.02† | (Marcombe et al. 2009, Saavedra-Rodriguez et al. 2012) |
| AAEL006805 | *CYP9J2* | 12.17 ± 2.83† | |
| AAEL006798 | *CYP9J10* | 2.89 ± 0.29† | (Strode et al. 2008, Bariami et al. 2012) |
| AAEL006814 | *CYP9J20v1* | 3.29 ± 0.20† | |
| AAEL014612 | *CYP9J21* | 3.94 ± 0.42† | |
| AAEL014609 | *CYP9J26* | 2.76 ± 0.22† | (Strode et al. 2008, Bariami et al. 2012) |
| AAEL014616 | *CYP9J27* | 2.85 ± 0.13† | (Strode et al. 2008, Bariami et al. 2012) |
| AAEL002633 | *CYP9J31* | 2.30 ± 0.07† | |
| AAEL013556 | *CYP4J15v1* | 2.30 ± 0.06† | (Marcombe et al. 2009†) |
| AAEL014829 | *CYP4J15v2* | 3.85 ± 0.22† | (Marcombe et al. 2009†) |
| AAEL007815 | *CYP4D24* | 2.81 ± 0.09† | |
| AAEL008345 | *CYP4G35* | 3.03 ± 0.08† | |
| AAEL006824 | *CYP4G35* | 3.44 ± 0.30† | |
| AAEL007830 | *CYP4H29* | 2.52 ± 0.18† | |
| AAEL003399 | *CYP4H30* | 3.85 ± 0.18† | |
| AAEL013798 | *CYP4H33* | 2.73 ± 0.03† | |
| AAEL004054 | *CYP4G36* | 2.84 ± 0.23† | (Saavedra-Rodriguez et al. 2012) |
| AAEL012766 | *CYP325G2* | 2.06 ± 0.04 | |
| AAEL012772 | *CYP325G3* | 3.61 ± 0.20† | |
| AAEL011769 | *CYP325M4* | 2.72 ± 0.11† | |
| AAEL002005 | *CYP12F6* | 2.14 ± 0.06* | (Strode et al. 2008) |
| AAEL006827 | *CYP12F8* | 2.30 ± 0.06† | |
| AAEL009119 | *CYP6N11* | 0.33 ± 0.01‡ | |
| AAEL009131 | *CYP6Z8* | 0.06 ± 0.01‡ | |
| AAEL006784 | *CYP9J17* | 0.31 ± 0.06‡ | |
| AAEL014611 | *CYP9J19* | 0.04 ± 0.01‡ | |
| AAEL014610 | *CYP9J29* | 0.39 ± 0.07‡ | |
| AAEL003748 | *CYP9AE1* | 0.38 ± 0.08‡ | |
| AAEL007812 | *CYP4H32* | 0.36 ± 0.02‡ | |
| AAEL014019 | *CYP4J17* | 0.44 ± 0.03‡ | |
| AAEL012765 | *CYP325M3* | 0.31 ± 0.02‡ | |

[a] Nomenclature for the cytochrome P450 genes was taken for the *Ae. aegypti* cytochrome P450 database at: http://drnelson.uthsc.edu/CytochromeP450.html.

[b] Vectorbase *Ae. aegypti* predicted gene set v. AaegL1.1. http://aaegypti.vectorbase.org/.

* Significantly up-regulated (more than twofold) at the $P < 0.05$ level of significance.

† Significantly up-regulated (more than twofold) at the $P < 0.01$ level of significance.

‡ Significantly down-regulated (more than twofold) at the $P < 0.01$ level of significance.

guez et al. 2012). Of the 33 up-regulated P450 genes, the following 13 were also reported in literature: *CYP6BB2*, *CYP6CB1*, *CYP6M1*, *CYP6N9*, *CYP6N12*, *CYP6Z6*, *CYP9J10*, *CYP9J26*, *CYP9J27*, *CYP4J15v1*, *CYP4J15v2*, *CYP4G36*, and *CYP12F6* (Table 4). Multiple studies have investigated the functional role of insect cytochrome P450s (Joussen et al. 2008; Müller et al. 2008, 2011;Zhu et al. 2010; Stevenson et al. 2011, 2012; Yang and Liu, 2011; Chandor-Proust et al. 2013), especially on cytochrome P450s in families CYP6 and CYP9. For example, Stevenson et al. (2012) investigated the function of seven family six and nine cytochrome P450s in *Ae. aegypti*, while multiple other studies have investigated family six and nine in other mosquito species as well (Boonsuepsakul et al. 2008, Duangkaew et al. 2011, Lertkiatmongkol et al. 2011). However, information on the functional role of cytochrome P450s family four in pyrethroid resistance is scarce. Therefore, four genes (*CYP4D24*, *CYP4H29*, *CYP4J15v1*, and *CYP4H33*) from family CYP4 were selected for further functional studies, of which only *CYP4J15v1* has been previously reported to be up-regulated in permethrin-resistant *Ae. aegpyti* (Marcombe et al. 2009), whereas the other three family four P450s have not been previously linked to insecticide resistance in *Ae. aegypti*. After successful full length cloning (Fig. 1) and sequence confirming, the four P450 genes from the Puerto Rico strain of *Ae. aegypti* were transferred and expressed in *D. melanogaster* under control of the GAL4–UAS enhancer trap system. When adult female *D. melanogaster* were exposed to permethrin at a concentration of 5 μg per vial, none of the control *D. melanogaster* (empty vector nontransgenic control) survived. However, when exposed to permethrin at 5 μg per

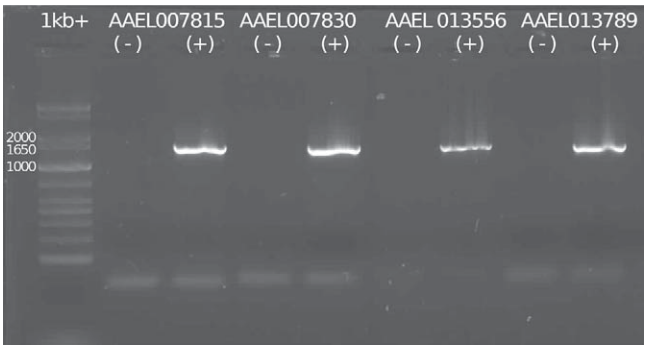


**Fig. 1.** RT-PCR of transgenic *D. melanogaster* expressing *Ae. aegypti* cytochrome P450 genes. The (−) and (+) within gene represent the amplified products from the nontransgenic empty vector control line (−) and the transgenic line (+) of *D. melanogaster*, respectively.

vial, transgenic *D. melanogaster* expressing *CYP4D24*, *CYP4H29*, *CYP4J15v1*, and *CYP4H33* had a survival rate of 60.0 ± 6.7, 29.0 ± 4.4, 64.4 ± 9.7, and 11.0 ± 4.4%, respectively (Fig. 2). When exposed to permethrin at a higher concentration (10 μg per vial), none of the control *D. melanogaster* survived. Similarly, none of the transgenic *D. melanogaster* expressing *CYP4J15v1* or *CYP4H33* survived when they were exposed to permethrin at 10 μg per vial. However, transgenic *D. melanogaster* expressing *CYP4D24* and *CYP4H29* had a survival rate of 37.8 ± 4.4 and 2.2 ± 2.2%, respectively (Fig. 2). Taken together, these results suggest that all these four P450s play some roles in the resistance of the Puerto Rico strain to permethrin, with *CYP4D24* playing a bigger role than the other three family 4 P450s used in this study. However, the fact that transgenic *D. melanogaster* expressing *CYP4D24*, *CYP4H29*, *CYP4J15v1*, and *CYP4H33* had a significantly higher survival rate when exposed to permethrin at 5 μg per vial compared with that when exposed to permethrin

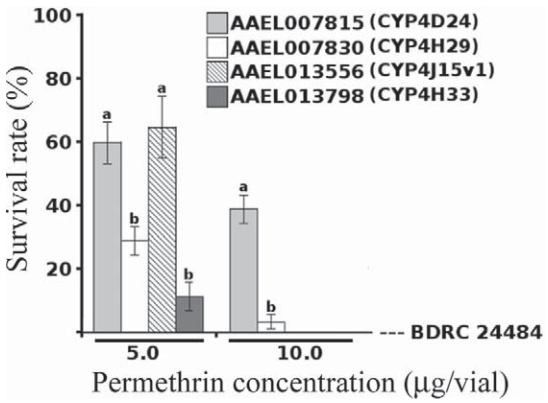


**Fig. 2.** Survival rate of transgenic *D. melanogaster* lines following a 24 h exposure to permethrin. Bars within dose superseded by the same letter are not significantly different ($P > 0.05$) whereas bars with different letter are significantly different ($P < 0.05$). BDRC 2,4484 is the nontransgenic empty vector control line of *D. melanogaster*, which had no survival after exposure to the two concentrations of permethrin used in this study.

at 10 μg per vial, suggesting that a single P450 gene might only play a partial role in the resistance to permethrin.

In conclusion, topical application bioassay revealed that the Puerto Rico strain of *Ae. aegypti* was 73-fold resistant to permethrin compared with the Orlando strain. In the presence of the cytochrome P450's inhibitor PBO, the resistance of Puerto Rico strain of *Ae. aegypti* was reduced to 15-fold, suggesting that cytochrome P450-mediated detoxification mechanism is involved in the resistance of the Puerto Rico strain of *Ae. aegypti* to permethrin. Of the 164 selected cytochrome P450s, 33 were significantly up-regulated more than twofold. Functional studies using *D. melanogaster* as a model insect, four family 4 cytochrome P450s selected for this study were found to confer some resistance to permethrin. When exposed to 5 μg per vial permethrin, transgenic *D. melanogaster* expressing *CYP4D24*, *CYP4H29*, *CYP4J15v1*, and *CYP4H33* had a survival rate of 60.0 ± 6.7, 29.0 ± 4.4, 64.4 ± 9.7, and 11.0 ± 4.4%, respectively. However, none of the control flies survived the permethrin exposure at the same concentration. Similarly, none of the transgenic *D. melanogaster* expressing *CYP4J15v1* or *4H33* survived when they were exposed to permethrin at 10 μg per vial. However, transgenic *D. melanogaster* expressing *CYP4D24* and *CYP4H29* had a survival rate of 37.8 ± 4.4 and 2.2 ± 2.2%, respectively. Taken together, our results suggest that *CYP4D24* might play an important role in cytochrome P450-mediated resistance to permethrin.

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## References Cited

**Allan, S. 2011.** Susceptibility of adult mosquitoes to insecticides in aqueous sucrose baits. J. Vector Ecol. 36: 59–67.

**Ashburner, M., K. G. Golic, and R. S. Hawley. 2005.** *Drosophila:* a laboratory handbook. Cold Spring Harbor Laboratory Press, Woodbury, NY.

**Bariami, V., C. M. Jones, R. Poupardin, J. Vontas, and H. Ranson. 2012.** Gene amplification, ABC transporters and Cytochrome P450s: unraveling the molecular basis of Pyrethroid resistance in the dengue vector, *Aedes aegypti*. PLoS Negl. Trop. Dis. 6: e1692. (doi:10.1371/journal.pntd.0001692).

**Bateman, J. R., A. M. Lee, and C. Wu. 2006.** Site-specific transformation of Drosophila via phiC31 integrase-mediated cassette exchange. Genetics 173:769–77. (doi:10.1534/genetics.106.056945).

**Bischof, J., R. K. Maeda, M. Hediger, F. Karch, and K. Basler. 2007.** An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases. Proc. Natl. Acad. Sci. U.S.A. 104: 3312–3317. (doi:10.1073/pnas.0611511104).

**Boonsuepsakul, S., E. Luepromchai, and P. Rongnoparut. 2008.** Characterization of *Anopheles minimus* CYP6AA3 expressed in a recombinant baculovirus system. Arch. Insect Biochem. Physiol. 69: 13–21. (doi:10.1002/arch.20248).

**Brand, A. H., and N. Perrimon. 1993.** Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118: 401–415.

**Chandor-Proust, A., J. Bibby, M. Régent-Kloeckner, J. Roux, E. Guittard-Crilat, R. Poupardin, M. A. Riaz, M. Paine, C. Dauphin-Villemant, S. Reynaud, et al. 2013.** The central role of mosquito in insecticide detoxification revealed by functional expression and structural modelling. Biochem. J. cytochrome P450 CYP6Zs 455: 75–85.

**Clark, G. G., U. R. Bernier, S. A. Allan, D. L. Kline, and F. V. Golden. 2011.** Changes in host-seeking behavior of Puerto Rican *Aedes aegypti* after colonization. J. Med. Entomol. 48: 533–537. (doi:10.1603/ME10207).

**Curtis, C. F., J. E. Miller, M. H. Hodjati, J. H. Kolaczinski, and I. Kasumba. 1998.** Can anything be done to maintain the effectiveness of pyrethroid-impregnated bednets against malaria vectors? Phil. Trans. R. Soc. London B 353: 1769–1775.

**Feyereisen, R. 1995.** Molecular biology of insecticide resistance. Toxicol. Lett. 82/83: 83–90.

**Ffrench-Constant, R. H., P. J. Daborn, and G. Le Goff. 2004.** The genetics and genomics of insecticide resistance. Trends Genet. 20: 163–170.

**Fonseca-Gonzalez, I., M. L. Quinones, A. Lenhart, and W. G. Brogdon. 2011.** Insecticide resistance status of *Aedes aegypti* (L.) from Colombia. Pest Manag. Sci. 67: 430–437.

**Gong, Y., T. Li, L. Zhang, X. Gao, and N. Liu. 2013.** Permethrin induction of multiple cytochrome P450 genes in insecticide resistant mosquitoes, *Culex quinquefasciatus*. Int. J. Biol. Sci. 9: 863–871.

**Gubler, D. J. 1988.** Dengue, pp. 223–260. *In* T Monarth (ed.), The Arboviruses, vol. 2. Epidemiology and Ecology. CRC, Boca Raton, FL.

**Gubler, D. J., and G. G. Clark. 1995.** Dengue/dengue hemorrhagic fever: the emergence of a global health problem. Emerg. Infect. Dis. 1: 55–57.

**Hemingway, J., N. J. Hawkes, L. McCarroll, and H. Ranson. 2004.** The molecular basis of insecticide resistance in mosquitoes. Insect Biochem. Mol. Biol. 34: 653–665.

**Hougard, J. M., S. Duchon, M. Zaim, and P. Guillet. 2002.** Bifenthrin: a useful pyrethroid insecticide for treatment of mosquito nets. J. Med. Entomol. 39: 526–533.

**Joussen, N., D. G. Heckel, M. Haas, I. Schuphan, and B. Schmidt. 2008.** Metabolism of imidacloprid and DDT by P450 CYP6G1 expressed in cell cultures of *Nicotiana tabacum* suggests detoxification of these insecticides in Cyp6g1 overexpressing strains of *Drosophila melanogaster*, leading to resistance. Pest Manag. Sci. 64: 65–73.

**Juntarajumnong, W., S. Pimnon, M. J. Bangs, K. Thanispong, and T. Chareonviriyaphap. 2012.** Discriminating lethal concentrations and efficacy of six pyrethroids for control of *Aedes aegypti* in Thailand. J. Am. Mosq. Control Assoc. 28: 30–37.

**Lertkiatmongkol, P., E. Jenwitheesuk, and P. Rongnoparut. 2011.** Homology modeling of mosquito cytochrome P450 enzymes involved in pyrethroid metabolism: insights into differences in substrate selectivity. BMC Res. Notes 4: 321.

**Liu, N. 2008.** Insecticide resistance in mosquitoes: development and mechanisms, pp. 75–91. *In* N. Liu (ed.), Recent Advances in Insect Physiology, Toxicology and Molecular Biology. Research Signpost, Kerala, India.

**Livak, K. J., and T. D. Schmittgen. 2001.** Analysis of relative gene expression data using real-time quantitative PCR and the $2^{(-\Delta\Delta Ct)}$ Method. Methods 25: 402–408.

**Manda H, P. Shah, S. Polsomboon, T. Chareonviriyaphap, F. Castro-Llanos, A. Morrison, R. G. Burrus, J. P. Greico, and N. L. Achee. 2013.** Contact irritant responses of *Aedes aegypti* using sublethal concentration and focal application of pyrethroid chemicals. PLoS Negl. Trop. Dis. 7: e2074. (doi:10.1371/journal.pntd.0002074).

**Marcombe, S., R. Poupardin, F. Darriet, S. Reynaud, J. Bonnet, C. Strode, C. Brengues, A. Yébakima, H. Ranson, V. Corbel, et al. 2009.** Exploring the molecular basis of insecticide resistance in the dengue vector *Aedes aegypti:* a case study in Martinique Island (French West Indies). BMC Genomics 10: 494. (doi:10.1186/1471-2164-10-494).

**McLaughlin, L. A., U. Niazi, J. Bibby, J.-P. David, J. Vontas, J. Hemingway, H. Ranson, M. J. Sutcliffe, and M.J.I. Paine. 2008.** Characterization of inhibitors and substrates of *Anopheles gambiae* CYP6Z2. Insect Mol. Biol. 17: 125–135.

**Müller, P., E. Warr, B. J. Stevenson, P. M. Pignatelli, J. C. Morgan, A. Steven, A. E. Yawson, S. N. Mitchell, H. Ranson, J. Hemingway, et al. 2008.** Field-caught permethrin-resistant *Anopheles gambiae* overexpress CYP6P3, a P450 that metabolises pyrethroids. PLoS Genet. 4:e1000286.

**Poupardin, R., M. Riaz, J. Vontas, J. P. David, and S. Reynaud. 2010.** Transcription profiling of eleven cytochrome P450s potentially involved in xenobiotic metabolism in the mosquito *Aedes aegypti*. Insect Mol. Biol. 19: 185–193. (doi:10.1111/j.1365–2583.2009.00967).

**Pridgeon, J. W., K. M. Meepagala, J. J. Becnel, G. G. Clark, R. M. Pereira, and K. J. Linthicum. 2007.** Structure-activity relationships of 33 piperidines as toxicants against female adults of *Aedes aegypti* (Diptera: Culicidae). J. Med. Entomol. 44: 263–269.

**Pridgeon, J. W., J. J. Becnel, G. G. Clark, and K. J. Linthicum. 2009.** Permethrin induces overexpression of multiple genes in *Aedes aegypti*. J. Med. Entomol. 46: 580–587.

**R Core Team. 2013.** R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. (http://www.R-project.org/).

**Reid, W. R., L. Zhang, F. Liu, and N. Liu. 2012.** The transcriptome profile of the mosquito *Culex quinquefasciatus* following Permethrin selection. PLoS ONE 7: e47163. (doi:10.1371/journal.pone.0047163).

**Saavedra-Rodriguez, K., A. F. Suarez, I. F. Salas, C. Strode, H. Ranson, J. Hemingway, and W. C. Black. 2012.** Transcription of detoxification genes after permethrin selection in the mosquito *Aedes aegypti*. Insect Mol. Biol. 21: 61–77.

**Stevenson, B. J., J. Bibby, P. Pignatelli, S. Muangnoicharoen, P. M. O'Neill, L. Y. Lian, P. Muller, D. Nikou, A. Steven, J. Hemingway, M. J. Sutcliffe, and M.J.I. Paine. 2011.** Cytochrome P450 6M2 from the malaria vector *Anopheles gam-*

*biae* metabolizes pyrethroids: sequential metabolism of deltamethrin revealed. Insect Biochem. Mol. Biol. 41: 492–502.

**Stevenson, B. J., P. Pignatelli, D. Nikou, and M. J. Paine. 2012.** Pinpointing P450s associated with pyrethroid metabolism in the dengue vector, *Aedes aegypti:* developing new tools to combat insecticide resistance. PLoS Negl. Trop. Dis. 6: e1595.

**Strode, C., C. S. Wondji, J.-P. David, N. J. Hawkes, N. Lumjuan, D. R. Nelson, D. R. Drane, S.H.P.P. Karunaratne, J. Hemingway, W. C. Black IV, et al. 2008.** Genomic analysis of detoxification genes in the mosquito *Aedes aegypti*. Insect Biochem. Mol. Biol. 38: 113–123.

**Strode, C., M. de Melo-Santos, T. Magalhães, A. Araújo, and C. Ayres. 2012.** Expression profile of genes during resistance reversal in a temephos selected strain of the dengue vector, *Aedes aegypti*. PLoS ONE 7: e39439. (doi:10.1371/journal.pone.0039439).

**Vulule, J. M., R. F. Beach, F. K. Atieli, J. M. Roberts, D. L. Mount, and R. W. Mwangi. 1994.** Reduced susceptibility of *Anopheles gambiae* to permethrin associated with the use of permethrin-impregnated bednets and curtains in Kenya. Med. Vet. Entomol. 8: 71–75.

**Warren, K. S., and A.A.F. Mahmoud. 1990.** Tropical and geographical medicine, 2nd ed. McGraw-Hill, New York, NY.

**Yang, T., and N. Liu. 2011.** Genome analysis of cytochrome P450s and their expression profiles in insecticide resistant mosquitoes, *Culex quinquefasciatus*. PLoS ONE 6: e29418. (doi:10.1371/journal.pone.0029418).

**Zhu, F., R. Parthasarathy, H. Bai, K. Woithe, M. Kaussmann, R. Nauen, D. A. Harrison, and S. R. Palli. 2010.** A brain-specific cytochrome P450 responsible for the majority of deltamethrin resistance in the QTC279 strain of *Tribolium castaneum*. Proc. Natl. Acad. Sci. U.S.A. 107: 8557–8562.

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